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Normal cells undergo apoptosis in response to inappropriate growth signals or the lack of overt survival signals. Tumor cells possess defects in apoptosis regulatory pathways and do not undergo apoptosis in these situations. There are two modes of apoptosis - an intrinsic pathway initiated by stress such as DNA damage and an extrinsic pathway resulting from activation of death receptors. Binding of ligand to a death receptor such as Fas, TNFR1 or TRAIL receptors 1 and 2 leads to activation of that receptor. This results in the recruitment of a cytoplasmic adaptor protein FADD to the receptor complex and activation of caspase-8. Because FADD is an essential component of receptor mediated apoptosis, a dominant-negative version (FADD-DN) is able to block both Fas and TNF induced apoptosis in many cell lines. However, experiments in our lab indicate that FADD-DN can kill normal human breast epithelial cells but not breast tumor cells. Since the only known role of FADD is an adaptor molecule, this suggests that FADD-DN interacts with one or more proteins expressed in breast epithelia. Because breast tumor cells do not die in response to FADD-DN, the potential FADD-DN interacting partners are likely to be involved in carcinogenesis.

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Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....4

Key Research Accomplishments.....6

Reportable Outcomes.....6

Conclusions.....7

References.....7

Appendices.....8

Introduction

Normal cells undergo apoptosis in response to inappropriate growth signals or the lack of overt survival signals. Tumor cells possess defects in apoptosis regulatory pathways and do not undergo apoptosis in these situations. There are two modes of apoptosis - an intrinsic pathway initiated by stress such as DNA damage and an extrinsic pathway resulting from activation of death receptors. Binding of ligand to a death receptor such as Fas, TNFR1 or TRAIL receptors 1 and 2 leads to activation of that receptor. This results in the recruitment of the cytoplasmic adaptor protein FADD to the receptor complex and activation of caspase-8. Because FADD is an essential component of receptor mediated apoptosis, a dominant-negative version (FADD-DN) is able to block both Fas and TNF induced apoptosis in many cell lines. However, experiments in our lab indicate that FADD-DN can kill normal human breast epithelial cells but not breast tumor cells. Since the only known role of FADD is an adaptor molecule, our hypothesis is that FADD-DN induces apoptosis in normal breast cells through interactions with one or more proteins expressed in breast epithelia. Our approach is to identify proteins that bind to FADD then identify the subset that are involved in FADD-DN binding using mutational analysis. Because breast tumor cells do not die in response to FADD-DN, the potential FADD-DN interacting partners are likely to be involved in carcinogenesis.

Body

We have achieved all the objectives described in the approved statement of work for year one and have made progress towards the objectives of year two. The scope of the first year of work was to identify FADD-DN binding partners expressed in normal breast cells. To accomplish this task we proposed to use the yeast two-hybrid system to identify FADD-DN binding partners then verify that these proteins were expressed in normal breast cells by Northern blotting and RT-PCR.

A two-hybrid screen was performed using FADD as the bait and a total of approximately 12 million clones were screened. The two-hybrid screen identified seventeen proteins as FADD binding partners: proteins with a known function include TRADD, RIP, DAXX, UBC9, SUMO, HoxD10, FHOS, KRAB, ROK1, ALEX, cAMP phospho-diesterase, MTA1 and SSDS. There were four proteins identified that have no assigned function: PL8, PL31, PL51, and PL78. We have likely identified all the possible FADD binding partners expressed in our cDNA library because we isolated most clones multiple times. Next, we determined which of these proteins are expressed in normal breast cells. Originally we proposed to do this by RT-PCR and Northern blotting. However, the CGAP database at NCI provided all the necessary information. This database lists the tissues that express a given sequence at the RNA level. All of our identified proteins are

expressed in normal breast cells as well as breast tumor cells. However we do not yet know the status of protein expression for our proteins.

We showed previously that RIP and TRADD are not involved in FADD-DN induced apoptosis (*1*). It is possible that many of the two-hybrid clones could be an artifact of yeast - they bind only in yeast or can activate two-hybrid reporters on their own. We therefore chose to perform *in-vivo* binding assays to determine which of the identified proteins bind to FADD in mammalian cells. Using this approach we determined that besides TRADD and RIP only PL31 binds to FADD in mammalian cells (see figure 1). We cannot exclude that other proteins may also bind but were not detected using this approach. For example PL78 is expressed at very low levels and therefore may be difficult to detect using immunoprecipitation. However we believe that PL31 is likely to be involved in FADD-DN induced apoptosis and will be discussed in further detail.

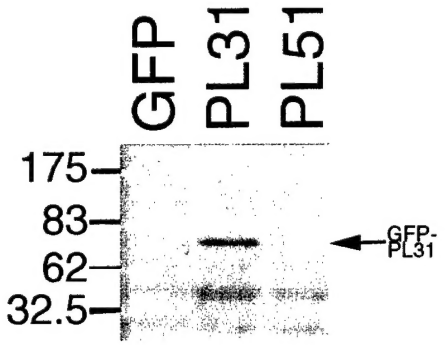

Figure 1: PL31 binds to FADD in mammalian cells. Two-hybrid proteins were tagged with GFP then transfected into HeLa cells stably expressing Flag tagged FADD. The Flag complex was immunoprecipitated using anti-Flag beads. The precipitate was eluted and expression of GFP fusions was determined by Western blotting.

In addition to PL31 we think that the TNF-related apoptosis inducing ligand (TRAIL) signaling pathway may be involved in FADD-DN induced apoptosis. The apoptotic pathway induced by FADD-DN can be inhibited by a broad caspase inhibitor (zVAD.fmk) when combined with a serine protease inhibitor (AEBSF). Alone neither is sufficient to inhibit death. Interestingly in normal prostate cells, TRAIL signaling can be inhibited by zVAD.fmk and AEBSF together, but not alone (*2*). There is much interest in TRAIL because reports indicate that it induces apoptosis in tumor but not normal cells (*3*). TRAIL binds to two signaling receptors (DR4 and DR5) as well as two decoy receptors (DcR1 and DcR2) (*4*). We have shown that DR4 and DR5 can bind directly to FADD and therefore are including these proteins in our study.

We assessed the ability of the GFP-tagged versions of the two-hybrid clones to kill normal breast cells. Interestingly GFP-PL31 killed normal breast cells in a manner similar to FADD-DN (see figure 2). Consistent with this, PL31 kills normal prostate cells but not prostate tumor cells. We also observed that a FLAG tagged version of PL31 transiently expressed in HeLa cells is cleaved in response to TRAIL. For these reasons and because PL31 binds to FADD in mammalian cells PL31 is our prime candidate as the

protein that binds to FADD-DN to induce apoptosis. We have ordered the production of a polyclonal PL31 antibody which will allow us to determine if PL31 is expressed at the protein level in normal breast cells as well as tumor cells.

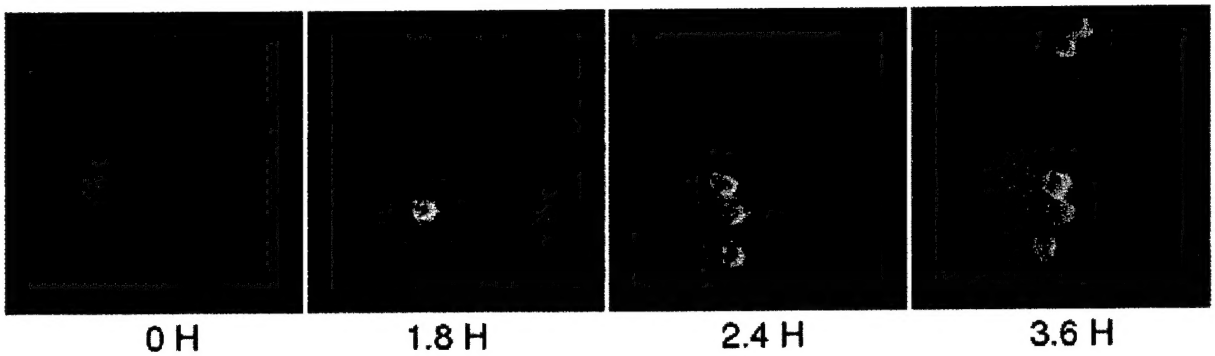

Figure 2: PL31 induces apoptosis in normal prostate cells. GFP tagged PL31 was microinjected into normal prostate cells. Green cells that express PL31were followed by time lapse microscopy. After 3.6 hours almost all the cells have undergone apoptosis.

Key Research Accomplishments

- •We identified 17 proteins that bind to FADD using a two-hybird screen.
- •It was determined that all of these proteins are expressed in normal breast cells.
- •Immunoprecipitation experiments showed that one of these proteins, PL31, binds to FADD in mammalian cells.
- •GFP-PL31 kills normal breast cells in a manner similar to FADD-DN and is our prime candidate.
- •A reverse two-hybrid system was developed. This system will allow us to identify mutations in FADD which is the scope of year two of this project.

Reportable Outcomes

Manuscripts:

L. R. Thomas, D. J. Stillman, A. Thorburn. Regulation of Fas-associated death domain interactions by the death effector domain identified by a modified reverse two-hybrid screen*J Biol Chem* 277, 34343-8 (Sep 13, 2002).

Abstracts:

June 2002: 18th Annual Meeting on Oncogenes "Lord of the Genes" *Regulation of FADD death domain interactions by the death effector domain identified by a modified reverse two-hybrid screen.*

February 2003: Keystone meeting "Molecular Mechanisms of Apoptosis" *Regulation of FADD death domain interactions by the death effector domain identified by a modified reverse two-hybrid screen.*

Conclusions

We identified 17 proteins that bind FADD using a two-hybrid screen. All of these clones were expressed in normal breast cells as determine using the CGAP database at NCBI. Using immunoprecipitation we determined that one clone, PL31, is able to bind to FADD in mammalian cells. In addition like FADD-DN, PL31 kills normal prostate and breast cells, but does not kill tumor cells. For these reasons we think it is likely that PL31 is involved in FADD-DN induce apoptosis. In addition we developed a reverse two-hybrid system which will allow us to perform mutational analysis in year two of this proposal.

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Regulation of Fas-associated Death Domain Interactions by the Death Effector Domain Identified by a Modified Reverse Two-hybrid Screen*

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The adapter protein FADD consists of two protein interaction domains and is an essential component of the death inducing signaling complex (DISC) that is formed by activated death receptors of the tumor necrosis factor (TNF) receptor family. The FADD death domain binds to activated receptors such as Fas or other adapters such as TRADD, whereas the FADD death effector domain binds to procaspase 8. Each domain can interact with its target in the absence of the other domain, and this has led to the idea that the two domains function independently. FADD death domain interactions with Fas and TRADD are thought to occur on the same surface; however, the regulation of these interactions is poorly understood. We developed a modified reverse two-hybrid method that can identify mutations, which inhibit some protein-protein interactions without affecting other interactions. Using this method, we identified mutations in FADD that prevent binding to Fas but do not affect binding to TRADD. Surprisingly, these mutations were in the death effector domain rather than the death domain. To test whether the mutants function in mammalian cells, we expressed wild type or mutant FADD molecules in FADD-deficient cells. Wild type FADD rescued both Fas ligand- and TNF-dependent signaling, whereas the FADD death effector domain mutants rescued only TNF signaling. These data indicate that in contrast to current models, the death effector domain of FADD is involved in interaction with Fas.

The six identified death receptors of the TNFR[1] family induce apoptosis by forming a complex called the DISC with intracellular proteins (1). Procaspase 8 is then cleaved and activated through interactions with various proteins leading to apoptosis. The adapter protein FADD (2) consists of two protein interaction domains. FADD binds to receptors or other adapters through its death domain and binds to procaspase 8 through its death effector domain. The recruitment of procaspase 8 to the DISC is thought to result in the autoactivation of the caspase. FADD binds directly to Fas to activate caspase 8 in response to Fas ligand and binds the adapter protein TRADD to activate caspase 8 in response to TNFα (1). Thus, the important interaction for caspase activation by Fas is between FADD and Fas death domains, whereas the corresponding interaction responsible for TNFα-induced caspase activation is between FADD and TRADD death domains. The solution structures of the death domain (amino acids 96–208) and the death effector domain (amino acids 1–81) of FADD have been solved (3–5). Both domains are globular structures consisting of six α-helices that are tethered together by a linker, which is thought to be flexible because it is sensitive to proteases (3). These studies suggest that binding between death domains occurs through charge interactions. By contrast, binding between the death effector domains of FADD and procaspase 8 is the result of hydrophobic interactions (6). Site-directed mutagenesis experiments suggest that the Fas-FADD and TRADD-FADD interactions occur on the same surface of the FADD death domain. Indeed, the mutations in helices 2 and 3 of the FADD death domain abolish interactions with both Fas and TRADD, although one mutation, FADD (R117A), seems to prevent binding to Fas only (7). Current models (3, 4, 8) are based on the idea that the two domains function independently of each other (*i.e.* that the death domain does not affect death effector domain interactions and *vice versa*). This view is supported by experiments showing that each domain in isolation can interact with its partner. For example, the isolated death domain can inhibit apoptosis by binding to activated Fas (8). However, the mechanisms through which FADD interactions are regulated in the context of the full-length protein are incompletely understood.

Reverse two-hybrid screens identify mutations in proteins that result in a loss of protein-protein interactions (10, 11). In this method, one typically uses *in vitro* mutagenesis to create a library of mutants of one of the components in a two-hybrid screen, either the DNA-binding domain (DBD) fusion plasmid or the activation domain (AD) fusion plasmid. The investigator then screens for the loss of two-hybrid interaction. The major problem with current reverse two-hybrid methods is that one commonly identifies mutations that prevent stable expression of the two-hybrid protein or that affect gross protein folding. Such mutants are often not useful for mechanistic studies. To help dissect FADD signaling, we developed a reverse two-hybrid system that identifies mutations, which specifically abolish interactions among particular partner proteins while requiring that the mutated protein still interacts with a different protein partner; thus, demanding that the mutant protein

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[1] The abbreviations used are: TNFR, tumor necrosis factor receptor; TNF, tumor necrosis factor; DISC, death-inducing signaling complex; FADD, Fas-associated death domain; TRADD, TNF receptor-associated death domain; AD activation domain; DBD, DNA-binding domain; Puro, puromycin; FasL, Fas ligand; GFP, green fluorescent protein.

is stably expressed in its native conformation. Using this method, we identified mutations in FADD, which suggest that in contrast to current models, the FADD death effector domain regulates the interaction between FADD and Fas.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The GAL1-TetR expression cassette was constructed by cloning the Tn10 Tet Repressor with two copies of the SV40 nuclear localization signal (one at the N-terminal and one C-terminal) between the GAL1 promoter and the ADH1 terminator. This GAL1-TetR expression cassette was then cloned into plasmid HO-poly-KanMX4-HO (9), which allows it to be integrated into the HO locus. To construct the TetO-ADE2 reporter, the promoter of the ADE2 gene was deleted and replaced with a promoter fragment containing TetO sites made by PCR amplification from strain YI584 (Bio101, Carlsbad, CA). Plasmid Gal4-DBD-(YIp-TRP1) was constructed by inserting a cassette with the ADH1 promoter, Gal4 DNA-binding domain, and ADH1 terminator from plasmid pAS2–1 (10) into pRS304 (11). Plasmid LexA-DBD-(YIp-URA3) was constructed by first inserting the ADH1 promoter Gal4 DBD-ADH1 terminator cassette into plasmid pRS306 (11) and then replacing the Gal4 DBD with an *Eco*RI-*Hin*dIII fragment from pBTM116 (12) containing the LexA DBD. Plasmid pACT3, a YEp vector with a LEU2 marker, was constructed by modifying the polylinker of the pACT2 activation domain vector (CLONTECH, Palo Alto, CA). Plasmid Gal4-DBD-FAS containing amino acids 191–313 of human Fas was made by PCR amplifying nucleotides 571–939 of human Fas and inserting it into the *Eco*RI and *Bam*HI sites of Gal4-DBD-(YIp-TRP1). The full-length TRADD cDNA was inserted into LexA-DBD-(YIp-URA3) at the *Eco*RI and *Bam*HI sites to give LexA-DBD-TRADD. For pACT3-FADD, a PCR fragment with full-length FADD was inserted into the *Eco*RI and *Bam*HI sites of pACT3. pCDNA3.1 Puro was constructed by replacing the Hygro gene of pCDNA3.1(+) Hygro with the Puro gene. FADD and FADD mutant cDNAs were cut from the pACT3 versions with *Eco*RI and *Xho*I and inserted into the same sites of pCDNA3.1 Puro. A detailed description of plasmid construction, maps, and sequences is available upon request.

Yeast Strains, Transformations, and Media—The genotype of SFY526 (13) is *MAT***a** *ade2 can1 his3 leu2 lys2 trp1 gal4 gal80 URA3*::*GAL1-lacZ*. The genotype of DY6877 is *MAT***a** *ade2 can1 his3 leu2 lys2 trp1 URA3*::*LexA(op)-lacZ*. The genotype of LY26 is *MATα can1 his3 leu2 met15 trp1 ura3 gal4*::*hisG gal80*::*hisG LYS2*::*LexA(op)-HIS3 TetO-ADE2 ho*::*KanMX*::*GAL1-TetR*. The *GAL80* gene in DY7088 (*ade2 can1 his3 leu2 met15 trp1 ura3 LexA(op)-HIS3*) was disrupted with a *gal80*::*hisG-URA3-hisG* disrupter and converted to *gal80*::*hisG* after growth on 5-fluoroorotic acid (14). The endogenous *ade2* gene was next replaced with the *TetO-ADE2* reporter, and the *KanMX*::*GAL1-TetR* reporter was integrated at the *ho* locus. Finally, *GAL4* was knocked out with a *gal4*::*hisG-URA3-hisG* disrupter and then converted to *gal4*::*hisG*. A detailed description of LY26 construction is available upon request. The medium was prepared as described previously (15). Transformations were done using the high efficiency lithium acetate method (16).

Reverse Two-hybrid Screen—The LY30 strain used in screening for FADD mutations was a derivative of LY26 with plasmids Gal4-DBD-FAS and LexA-DBD-TRADD integrated at the *TRP1* and *URA3* loci, respectively. To generate a randomly mutagenized library, FADD cDNA was PCR-amplified from pACT3-FADD using primers GAD-20 (5′-CGATGATGAAGATACCCCACC-3′) and ACT+40 (5′-ATGGTG-CACGATGCACAG-3′) in the presence of 50 μM $MnCl_2$ (17). The reactions were ethanol-precipitated then cotransformed with 1 μg of pACT3 linearized with *Eco*RI into LY30 using gap repair to create circular plasmids by recombination in yeast. Transformants were selected on −Trp−Leu−Ade−His + 1 mM 3-amino triazole plates (−Trp decreases background by retaining Gal4-DBD-FAS, and -Leu selects the prey plasmid). After 3 days, colonies were mated to SFY526 or DY6877. Diploids were selected on −Trp−Leu−Met media and tested for lacZ activity. True positives showing strong binding phenotypes were rescued into *Escherichia coli* and then reintroduced into yeast to verify the phenotype.

Screen for Compensating Mutations—A mutagenized FADD (R71A) library was made as before using pACT3-FADD (R71A) as template. The screen was performed in yeast strain AH109 (*MAT***a** *trp1 leu2 his3 gal4 gal80 LYS2*::*GAL1-HIS3 GAL2-ADE2 URA3*::*MEL1-lacZ* CLONTECH, Palo Alto, CA) expressing Gal4-DBD-Fas. Approximately one million FADD (R71A) molecules were screened, and positive two-hybrid interaction was selected for on -Trp-Leu-Ade-His + 1 mM 3-amino triazole medium. Seven clones showing strong LacZ activity were rescued into *E. coli* and then retested for two-hybrid interaction in strain SFY526.

Cell Lines and Apoptosis Assays—Jurkat cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum. The FADD-deficient Jurkat cells (18) were stably transfected with linearized pCDNA3.1 Puro expressing wild type or mutant FADD cDNAs by electroporation and selected with puromycin. For induction of apoptosis, 3 ml of Jurkat cells (7.5×10^5 cells/ml) were treated with 2.0 ng/ml FasL (Upstate Biotechnology, Lake Placid, NY) for 3 h or 25 ng/ml TNF (R&D Systems, Minneapolis, MN) in the presence of 1 μg/ml cycloheximide for 3 h. Primary antibodies for Western blots were FADD (BD Transduction Laboratories), caspase 8, caspase 3 (Cell Signaling, Beverly, MA), and β-actin (Sigma). Fas DISC immunoprecipitations were performed using 100 million Jurkat cells for each sample. Cells were treated with or without FLAG-tagged Fas ligand (Alexis Biochemicals, San Diego, CA). Anti-FLAG antibody (Sigma) then was added to all samples, and DISC immunoprecipitations were performed as described previously (19). Caspase 3 assays were performed as described previously (20).

RESULTS

Reverse Two-hybrid Screening—We modified the yeast split hybrid system (21) to include reporters for two DBD fusion proteins (sometimes called "baits"), similar to dual bait systems (Fig. 1) (24, 25). The first bait fused to the Gal4 DNA-binding domain is used to detect loss of interaction via a dual reporter system. A two-hybrid interaction between the Gal4-DBD fusion and the AD fusion results in the expression of the Tn10 Tet repressor, which blocks transcription of *ADE2* from the *TetO-ADE2* reporter. Thus, the two-hybrid interaction with the Gal4-DBD fusion results in no *ADE2* expression and an Ade− phenotype. A mutation that disrupts this interaction removes *ADE2* inhibition, and the yeast are able to grow in the absence of adenine. Thus, we can select for the loss of two-hybrid interaction by selecting for Ade+ yeast. The second bait protein, a LexA-DBD fusion, is used to eliminate mutations in the AD fusion plasmid that affect expression or stability of the AD protein fusion. Two-hybrid activation between the prey and the LexA-DBD fusions will activate the *LexA(op)-HIS3* reporter, resulting in an His+ phenotype. Thus, specific mutations in the AD fusion that block interaction with partner 1 (the Gal4-DBD fusion) but maintain overall protein integrity, allowing interaction with partner 2 (the LexA-DBD fusion), can be selected as Ade+ His+ transformants (Fig. 1*C*). Importantly, the sensitivity of both two-hybrid interactions can be titrated either with tetracycline, which modulates the activity of the Tet repressor (21), or with 3-amino triazole, which increases the amount of *HIS3* expression required for histidine prototrophy (10). This allows the identification of strong and weak mutant alleles for both interactions.

To establish that the system works, plasmids with Gal4-DBD-FAS and LexA-DBD-TRADD were integrated into the yeast genome. Three activation domain plasmids, the empty AD vector, the AD-FADD (wild type) fusion, and the AD-FADD (R117A) mutant, were transformed and tested for two-hybrid interaction with Gal4-DBD-FAS and LexA-DBD-TRADD (Fig. 1*D*). When assayed on selective media, yeast carrying the empty AD vector do not activate the *LexA (op)-HIS3* reporter and display an His− phenotype. Similarly, yeast expressing the AD-FADD (wild type) fusion have two-hybrid activation of the *GAL1-TetR* gene, resulting in the repression of the *TetO-ADE2* reporter and an Ade− phenotype. The FADD (R117A) mutant binds to TRADD but is not able to bind to Fas *in vitro* (7) or in yeast (22). When tested in the dual bait two-hybrid strain, the AD-FADD (R117A) fusion interacts with LexA-DBD-TRADD, resulting in an His+ phenotype. Failure of AD-FADD (R117A) to interact with Gal4-DBD-FAS leads to no expression of TetR, resulting in ADE2 expression and an Ade+ phenotype. Thus, the differential

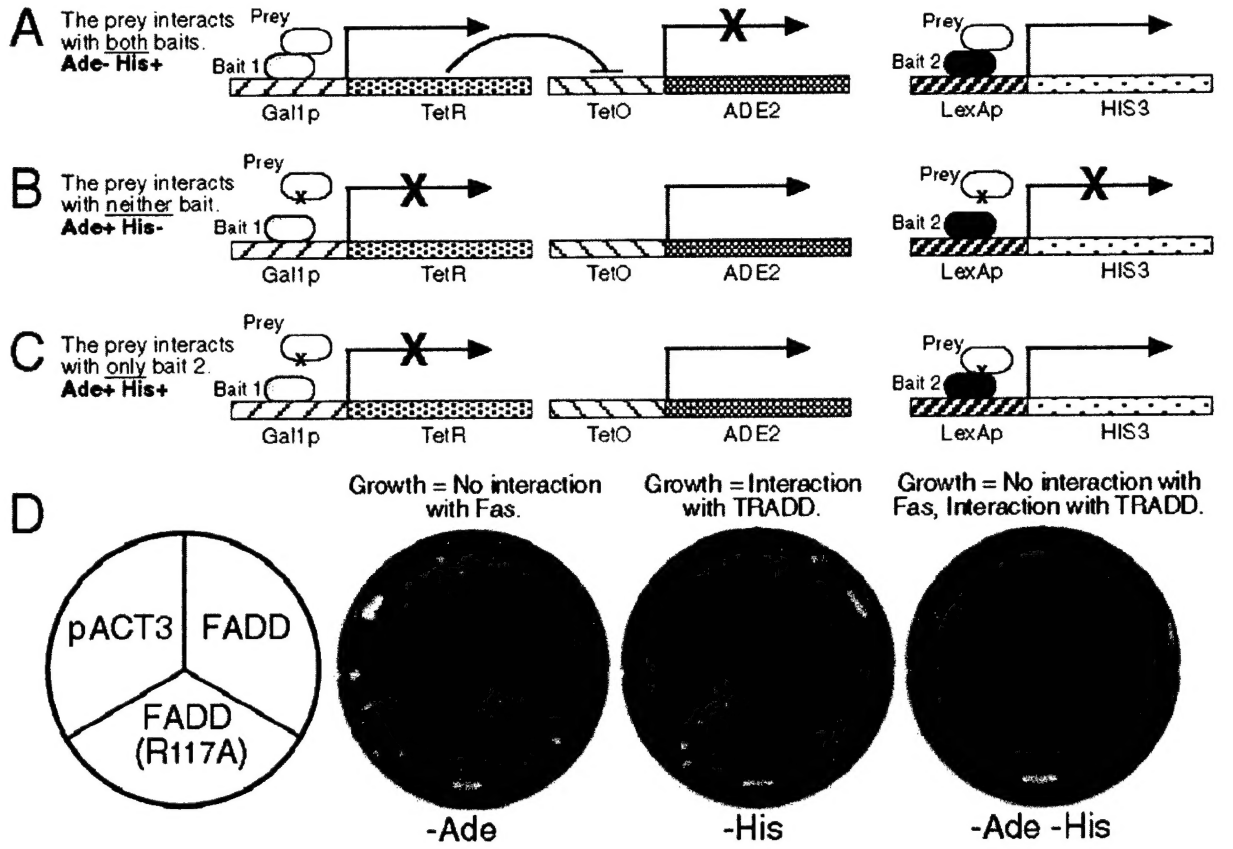

FIG. 1. **Reverse two-hybrid system to identify specific mutants.** *A*, binding of the prey to partner 1 fused to the Gal4 DNA-binding domain results in the transcription of TetR, which binds to TetO, shutting off transcription of ADE2, and yeast are not able to grow in the absence of adenine. Yeast are His+ if the prey interacts with bait 2. *B*, a mutation in the prey that prevents interaction with partner 1 results in ADE2 expression because TetR is not transcribed. Yeast expressing preys with such mutations can grow on medium lacking adenine. If this mutation compromises protein structure, HIS3 is not expressed. *C*, only mutations that are specific for loss of interaction with bait 1 result in both ADE2 and HIS3 expression. *D*, yeast expressing Gal4-DBD-FAS (partner 1) and LexA-DBD-TRADD (partner 2) were transformed with empty vector (pACT3), wild type FADD, or FADD (R117A). Yeast expressing the empty vector and the R117A mutant fail to interact with Fas and grow on −Ade medium, yeast expressing wild type FADD, and FADD (R117A) grow on −His media. Only yeast expressing FADD (R117A) can grow in the absence of both adenine and histidine.

interaction of the AD-FADD (R117A) mutant with the two baits results in an Ade+ His+ phenotype.

Identification of Mutations That Prevent FADD-Fas but Not FADD-TRADD Interactions—To identify other FADD mutants that discriminate between Fas and TRADD, a library consisting of the GAL4 activation domain fused to a randomly mutated FADD molecule was generated through mutagenic PCR followed by gap repair in yeast. A screen of 23,500 mutant FADD molecules yielded 120 Ade+ His+ transformants. These colonies potentially represent mutations in FADD that do not bind to Fas but retain interaction with TRADD. Each transformant was mated to a strain carrying either a *GAL1-lacZ* or *LexA-lacZ* reporter, and the two-hybrid activation of these reporters was used both to eliminate false positives and to determine the strength of each interaction. Several plasmids showing strong binding phenotypes were assayed for their ability to bind to a *GAL4*-caspase 8 bait to ensure that the mutation was specific for the loss of Fas binding only.

Plasmids encoding FADD molecules that show decreased binding to Fas but still bind to TRADD (Fig 2*A*) were sequenced. As the FADD-Fas interaction is mediated through death domain interactions, we expected to find mutations in the death domain of FADD. Surprisingly, the mutations mapped to the death effector domain (Fig. 2*B*). Histidine 59 was mutated to tyrosine (H59Y), arginine 71 was mutated to tryptophan (R71W), and arginine 72 was mutated to either cysteine (R72C) or histidine (R72H). These mutations are in surface amino acids that flank helix 5 of the FADD death effector domain (Fig. 2*C*). The mutations at positions 71 and 72 showed complete loss of binding to Fas, whereas the H59Y mutation showed intermediate levels as measured using a β-galactosidase reporter gene in the two-hybrid assay (Fig. 2*D*). All of the mutations bound to TRADD and caspase 8 about as well as the wild type FADD protein. We used site-directed mutagenesis to investigate other amino acid substitutions at these positions. When Arg-72 was changed to either an alanine (R72A) or a glutamate (R72E) and was tested for interaction with Fas in yeast, both mutations prevented interaction (Fig. 2*D*). However, when the same substitutions were made at position 71 (R71A and R71E), only a change to alanine prevented interaction. All of the mutations interacted with TRADD and caspase 8 similar to wild type FADD with the exception of the R72E mutation, which bound to TRADD only.

FADD Mutants Reconstitute TNFR Signaling but Not Fas Signaling—To test whether the mutations in FADD identified in yeast have a phenotype in mammalian cells, wild type FADD and two of the mutants (R71W and H59Y) isolated in yeast were expressed in FADD-deficient Jurkat I2.1 cells (23). To verify stable expression, cell extracts were Western blotted with an anti-FADD antibody. Cell lines transfected with FADD or FADD mutants but not green fluorescent protein (GFP) or the parental FADD-deficient cells (I2.1) had a unique band

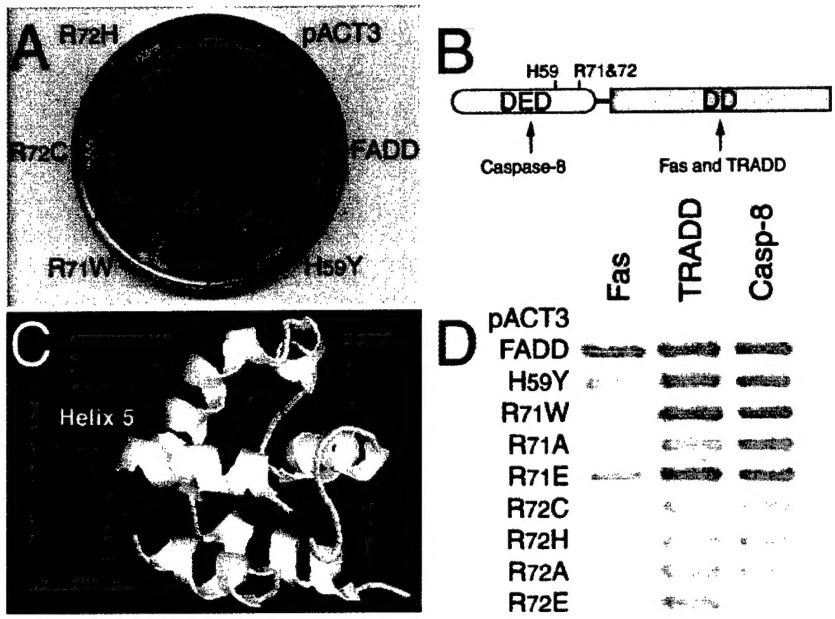

FIG. 2. **Mutations in FADD that do not bind Fas but still bind TRADD.** *A*, yeast expressing FADD mutants were grown on −Trp−Leu−Ade−His + 1 mM 3-amino triazole media. Yeast expressing FADD molecules with mutations at positions 59, 71, or 72 are able to grow on selective media, indicating loss of interaction with Fas but retention of interaction with TRADD. *B*, linear map of FADD with the location of mutations identified in our screen. *C*, NMR structure of the death effector domain of FADD (5). The three mutations that disrupt Fas binding are on either side of helix 5. *D*, empty vector, wild type FADD, or FADD mutants were tested for interaction with Fas, TRADD, or caspase 8 using a *LacZ* reporter in yeast.

with an apparent molecular mass of 26 kDa corresponding to FADD protein (Fig. 3*A*). The FADD mutants were expressed at similar levels, which were slightly lower than those of endogenous FADD in wild type Jurkat A3 cells. Cell lines expressing the FADD cDNAs with mutations at amino acid 72 produced very little FADD protein and were not examined further. A FADD mutant that can bind to TRADD but not Fas should reconstitute TNFR1 signaling but not Fas signaling in the I2.1 cells. Because the I2.1 cells were made by treating with a mutagen and selecting for growth in high levels of Fas ligand (23), they may contain other mutations that affect downstream Fas signaling. This appeared to be the case because even when we expressed wild type FADD in these cells, the cytotoxicity in response to Fas or TNFα was significantly reduced compared with the parental A3 cells (data not shown). Therefore, to test whether our mutant FADD molecules function as predicted in mammalian cells, we assayed caspase processing because this is the immediate downstream response caused by FADD binding to Fas or TRADD.

We treated the Jurkat cell lines with Fas ligand, which should result in the recruitment of FADD and activation of caspase 8 followed by activation of caspase 3. Wild type Jurkat A3 cells and cells expressing wild type FADD exhibited Fas ligand-dependent caspase 8 cleavage, whereas cells expressing GFP or R71W mutant version of FADD did not (Fig. 3*B*). The mutation from histidine to tyrosine at position 59 weakens but does not eliminate the interaction with Fas in yeast (Fig. 2*D*). Consistent with this finding, Jurkat cells expressing the H59Y mutant still showed some caspase 8 processing in response to Fas ligand. We next tested whether the mutants could mediate caspase 8 activation in response to TNFα. Caspase 8 was cleaved in wild type Jurkat cells and cells expressing wild type FADD and both FADD mutants but not in the parental I2.1 cells and the cells expressing GFP (Fig. 3*B*). Similar results were obtained when we monitored caspase 3 processing. TNFα treatment caused caspase 3 activation as determined by the appearance of the active cleaved form of the enzyme in parental cells and in FADD-deficient cells expressing wild type FADD or either of the point mutants. Conversely, caspase 3 processing in response to Fas ligand was observed only when wild type FADD was expressed. These data indicate that the FADD R71W and H59Y mutants are compromised in their ability to mediate Fas signaling but not TNFR signaling. This finding is consistent with the mutants retaining native conformation to allow interaction with TRADD but not with Fas.

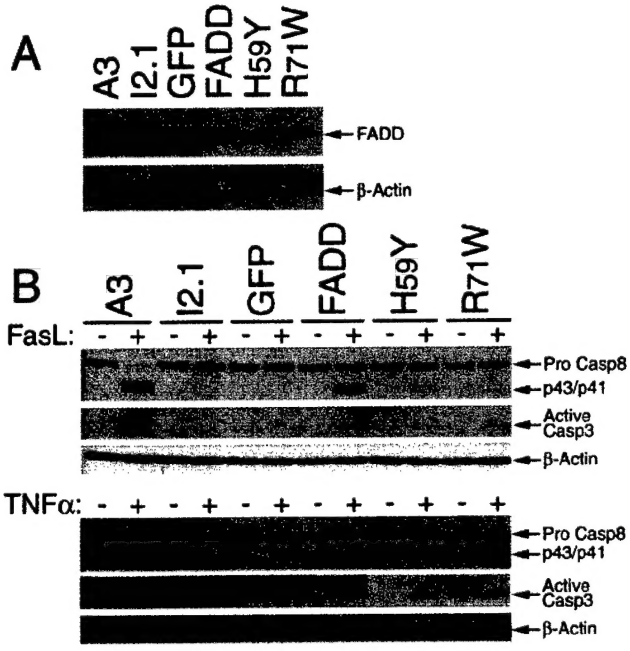

FIG. 3. **FADD mutants can reconstitute TNFR1 signaling but not Fas signaling.** *A*, wild type (*A3*), FADD-deficient (*I2.1*), or Jurkat cells stably expressing GFP, wild type FADD, or FADD mutants were Western blotted for FADD. *B*, protein samples from the same cells were probed with anti-caspase 8 or caspase 3 after treatment with or without Fas ligand or TNFα. Blots were stripped and reprobed with anti-β-actin to test for equal loading.

To directly test whether mutations in the FADD death effector domain prevented binding to Fas in mammalian cells, DISC immunoprecipitation assays were performed using I2.1 cells expressing GFP, wild type FADD, R71W, or H59Y mutants. We coprecipitated FADD at the Fas DISC in cells expressing wild type FADD but not cells expressing GFP or the R71W FADD mutant. Furthermore, consistent with the reduced binding of the H59Y mutant in yeast, a faint signal was observed with this mutant in DISC immunoprecipitates from Jurkat cells

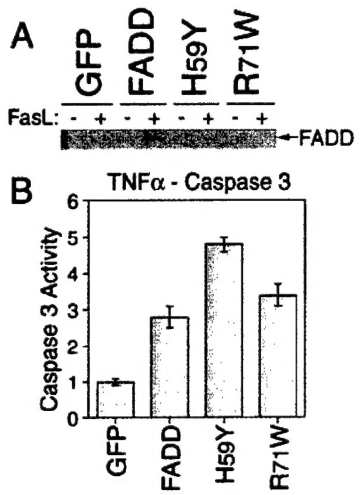

FIG. 4. **Mutations in FADD prevent binding to Fas in mammalian cells.** *A*, Jurkat cells stably expressing GFP, wild type FADD, or the Tyr-59 and Trp-71 FADD mutants were treated with cross-linked FLAG-FasL. The DISC was precipitated and Western blotted for FADD. *B*, Jurkat cells were treated with TNFα, and cell extracts containing 100 μg of protein were used to test caspase 3 activity using a colorimetric assay.

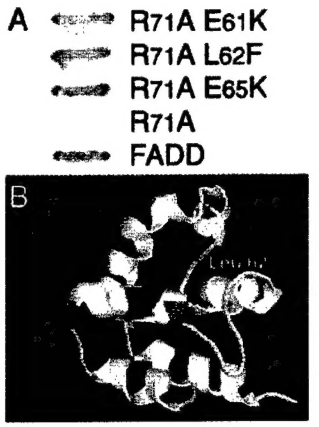

FIG. 5. **Second-site mutations that restore interaction with Fas are in helix 5 of the death effector domain.** *A*, yeast strain SFY526 was transformed with GAL4-FasΔ3 and the indicated FADD molecules; interaction with Fas was measured using a *lacZ* reporter. *B*, NMR structure of the death effector domain showing the location of mutations that restore binding of FADD (R71A) to Fas. Glu-61 and Glu-65 were changed to Lys, and Leu-62 was changed to Phe.

(Fig. 4*A*). The recruitment and activation of caspase 8 via interaction with FADD leads to caspase 3 activation. Therefore, we treated the Jurkat cell lines with TNFα to further test whether TRADD-dependent signaling via FADD was functional in these cells. Cells expressing wild type FADD and both FADD mutants but not cells expressing GFP displayed caspase 3 activity (Fig. 4*B*). The FADD mutants were as effective (R71W) or more effective (H59Y) than the wild type protein at activating caspase 3 in response to TNFα.

We next performed a screen for second-site mutations that compensate for loss of binding to Fas. We randomly mutagenized the R71A mutant, which cannot interact with Fas (Fig. 2*D*) and performed a forward two-hybrid screen to identify mutants that could now bind to Fas. Fig. 5*A* shows three second-site mutations, which allow the R71A mutant to bind to Fas as well as the wild type FADD protein. Interestingly, these mutations (E61K, L62F, and E65K) were in helix 5 of the death effector domain (Fig. 5*B*). Thus, a mutation in the loop between helices 5 and 6 of the death effector domain abolishes interaction between FADD and Fas, but further mutations in helix 5 rescue the interaction. These data further indicate that the death effector domain plays a role in mediating interaction between FADD and Fas and suggest that helix 5 is important in this response.

DISCUSSION

In this paper, we describe a modified reverse two-hybrid system that permits the facile identification of mutations that inhibit some protein-protein interactions without affecting overall protein structure or binding to other proteins. Using this approach, we identified mutations in the death effector domain of FADD that prevent binding to Fas but do not alter binding to TRADD. FADD mutants that were identified using the reverse two-hybrid system showed the expected ability to reconstitute TNFR1 signaling but not Fas signaling when they were expressed in FADD-deficient Jurkat cells. These data indicate that the binding characteristics in yeast were mirrored in mammalian cells. The fact that mutations in the death effector domain of FADD prevent interaction with Fas suggests that current models for the formation of the DISC (24) in which the FADD death domain functions independently of the death effector domain to bind Fas are oversimplified. Rather than functioning as two separate entities, we suggest that the death domain and the death effector domain of FADD work together to regulate Fas binding.

A simple explanation for our results is that the FADD death effector domain directly participates in binding to Fas. Alternative mechanisms include an inhibitory effect of the death effector domain that is promoted by our mutations or an allosteric modification of the death domain by the death effector domain. Our results do not conclusively discriminate between these possibilities. However, the fact that we rescued the binding of the R71A mutant by introducing new mutations in helix 5 of the death effector domain suggest that it is the death effector domain itself and not an effect by the death effector domain on the death domain that is important. By performing structural studies using mutants similar to those identified with our screening method, it may be possible to determine how the death effector domain regulates death domain interactions. The death effector domain of FADD may also be involved in interaction with the TRAIL receptors where it is thought that DAP3, which binds to the death effector domain, is the adapter responsible for FADD recruitment (25). Our data also suggest that it may be possible to design drugs that specifically interfere with some but not all FADD interactions by searching for molecules that disrupt death domain interactions through an effect on the death effector domain. The yeast system might be a useful screening method for such molecules, which could be used to selectively inhibit signaling by some death receptors without affecting signaling from the other receptors that use FADD.

The reverse two-hybrid system can be used to study any protein interactions that occur in yeast and should be generally useful for the identification of mutants that have specific protein binding characteristics. This may allow the generation of dominant negative mutants that selectively inhibit specific activities of a particular protein. In addition, the system may be generally useful for the identification of mutants that provide useful insights into the structural requirements for specific protein-protein interactions.

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